Diversity and functions of bacteria associated with springtails

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Chapter 4 – Antimicrobial activity in culturable gut microbial communities of springtails

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4.1. Abstract

Aims
The rise of antibiotic resistance pushes the pharmaceutical industry to continually search for substances with new structures and novel mechanisms of action. Many environmental niches are still to be explored as sources of antimicrobials. In this paper we assess the antimicrobial potential of gut microbes of springtails, soil invertebrates which live in a microbe-dominated environment and are known to be tolerant to entomopathogenic microorganisms.

Methods and Results
Bacteria isolated from the guts of five springtail species were tested for inhibitory activity against different microbial pathogens. We identified 46 unique isolates belonging to 17 genera and 15 families. Thirty-five of these isolates (76%) showed inhibitory activity, and 18 inhibited both bacterial and fungal pathogens. One isolate was active against all the pathogens tested.

Conclusions
We demonstrated a range of antimicrobial activities in bacteria isolated from the guts of springtails, indicative of complex interactions within the gut community, possibly relating to nutrition or defense against pathogens.

Significance and impact of the study
Our results suggest that a large proportion of cultivatable microbes associated with Collembola have a potential for antimicrobial production. We propose that soil invertebrates and their associated microbes are interesting targets for drug discovery.

4.2. Introduction

Various biologically active compounds obtained from plants, animals and microbes have been used as therapeutic agents in traditional and modern medicine (Zhang, Sun and Wang 2013; Kong and Tan 2015). Despite an increased focus on synthetic and semi-synthetic compounds in the past decades (Patridge *et al.* 2016), natural products and their derivatives are still important leads in drug development (Li and Vederas 2009), and several sources remain to be explored (Taylor 2013; Pidot *et al.* 2014). Most natural products are of microbial origin (Peláez 2006) and many are derived from actinomycetes (Actinobacteria, Actinomycetales), a group of prolific antibiotic producers (Genilloud *et al.* 2011). These bacteria can be isolated from a variety of terrestrial and marine environments (Gontang *et al.* 2010; Guo *et al.* 2015) and are also found in association with animal and plant hosts (Qin *et al.* 2011).

Invertebrates' guts constitute a habitat for distinctive communities of microorganisms (König 2006) and offer opportunities for the evolution of specific

and complex symbioses (Noda *et al.* 2009). Gut microbes can contribute to the health and fitness of their host by participating in important functions such as nutrition, regulation of metabolism, development, immunity and defense against pathogens (Engel and Moran 2013). Importantly, resident microbial communities may contribute to defense against pathogens by preventing foreign microbes from establishing in the host, a phenomenon called colonization resistance (He *et al.* 2014). Different mechanisms can drive colonization resistance: symbiotic bacteria can contribute indirectly to pathogen defense by modulating host immunity mechanisms (Dong, Manfredini and Dimopoulos 2009). Alternatively they can interfere directly with colonization by external microorganisms through resource competition (Maltby *et al.* 2013) or through interference competition, by producing antimicrobials targeting the invading microorganisms (Rea *et al.* 2010).

Host-associated microbes, whether symbiotic or entomopathogenic, have significant potential as sources of secondary metabolites of biotechnological interest (Bode 2009; Piel 2009). For example, a *Pseudomonas* symbiont of the beetle *Paederus fuscipes* produces pederin-type polyketides, which are used as chemical weapons by the host . These compounds were also shown to contain antitumor properties (Piel *et al.* 2005). In this context, microbial communities associated with Collembola (Hexapoda) deserve special attention.

Collembolans, commonly known as springtails, are microarthropods that are found in soil ecosystems throughout the world. Recently, it was discovered that beta-lactam biosynthesis genes are widespread among collembolan families, while they have not been found in other animals (Roelofs *et al.* 2013; Suring *et al.* 2017). These genes probably originated by horizontal gene transfer from microorganisms living in close proximity with the springtails (Faddeeva-Vakhrusheva *et al.* 2016). While the function of these genes is still to be elucidated, it is suggested that they are involved in mechanisms regulating the composition of the gut microbial communities (Thimm *et al.* 1998) and possibly protecting the springtails from invasion by pathogenic microorganisms. Previous studies have shown that *Beauveria bassiana* and other entomopathogenic fungi are generally of low virulence to springtails (Broza, Pereira and Stimac 2001; Dromph and Vestergaard 2002), but the mechanisms explaining this resistance have not yet been described. Symbiotic bacteria may contribute to host defense by producing antimicrobial compounds that inhibit pathogen growth. Therefore, evaluating the inhibitory activity of bacteria isolated from collembolans, resistant to entomopathogenic microbes, has ecological relevance, because it could suggest a possible mechanism to explain the non-susceptibility of springtails to these pathogens.

Here, we screened microbial communities associated with soil invertebrate guts for inhibitory activity against pathogens. First, we isolated cultivable bacteria from the guts of five springtail species belonging to three different families within the order

Entomobryomorpha: *Folsomia candida* and *F. fimetaria* (Isotomidae), *Orchesella cincta* and *Sinella curviseta* (Entomobryidae), and *Tomocerus minor* (Tomoceridae). To enrich the bacterial pool for potential antibiotic-producers, we used culture media targeting actinomycetes. The colonies were then genotyped and dereplicated using repetitive element sequence-based PCR (rep-PCR) and random amplified polymorphic DNA (RAPD) PCR and identified with 16S rRNA gene sequencing. Finally, biological activity of the isolates was assessed by testing their ability to inhibit bacterial, fungal and oomycete pathogens.

4.3. Materials and methods

Test organisms

The animals were kept in plastic boxes with a plaster of Paris bottom and reared under constant conditions in climate rooms with 20ºC air temperature, 75% humidity and a 12:12 light dark regime. *F. candida*, *F. fimetaria* and *S. curviseta* were fed dried baker's yeast (Dr. Oetker, Bielefeld, Germany); *T. minor* and *O. cincta* were fed algae growing on twigs of pine trees. *F. candida* ("Berlin strain", Vrije Universiteit Amsterdam) originated from a population that had been cultured in our laboratory for more than 10 years. The other springtail strains were sampled from different locations in the Netherlands and reared in the laboratory for several generations before conducting the experiments.

Isolation and growth of bacteria from springtails

Guts were dissected from adult springtails (ten individuals for *F. candida*, *F. fimetaria* and *S. curviseta*, five for *T. minor* and *O. cincta*) using sterile forceps and tweezers. Dry ice was used as a source of carbon dioxide to anesthetize the animals. Dissected guts were placed in sterile Phosphate Buffer Saline (PBS) and crushed using a sterile plastic pestle. Ten-fold dilutions of the original extract were prepared, until dilution factor 10^{-5}. 100 µl of the undiluted extract and of the four dilutions was spread on Actinomycete Isolation Agar (M490) medium (per liter: sodium propionate 4 g; sodium caseinate 2 g; dipotassium phosphate 0.5 g; L-asparagine 0.1 g; magnesium sulphate 0.1 g; ferrous sulphate 0.001 g). The procedure was repeated twice, obtaining ten plates per species. 100 µl of sterile PBS was used as a negative control. Plates were incubated at 30ºC and observed daily for bacterial growth. Colonies of different morphologies were selected, transferred individually to Tryptic Soy Agar (TSA) plates (Tryptic Soy Broth, agar 1.5%; Sigma-Aldrich, St. Louis, MO, USA) to obtain pure cultures and kept at 4ºC until further characterization and screening steps.

Genotyping of isolates

Rep-PCR, RAPD-PCR and 16S rRNA gene sequencing were used to characterize and dereplicate the pure cultures obtained from the animals' guts. Genomic DNA was isolated from bacterial colonies using the PowerSoil DNA Isolation Kit (MOBIO Laboratories Inc., Carlsbad, CA, USA). First, rep-PCR was used to identify unique strains, to avoid redundant screening. The strains with unique rep-PCR profiles and the ones that did not produce bands were identified using 16S rRNA gene sequencing. We then used RAPD-PCR to 1) characterize the isolates that were not amplified by the rep primers, 2) obtain clear banding patterns for the isolates that did not have very clear rep-PCR profiles, and 3) try to differentiate between isolates identified as the same species based on 16S rRNA gene sequencing, but obtained from different springtail species.

Repetitive element sequence-based PCR (rep-PCR) and random amplified polymorphic DNA (RAPD) PCR

The first screening, to identify unique strains, was performed using primers REP1R-I (5' - III ICG ICG ICA TCI GGC - 3') and REP2-I (5' - ICG ICT TAT CIG GCC TAC - 3') (Versalovic, Koeuth and Lupski 1991). The second screening, to differentiate isolates that were identified as the same genus by 16S rRNA gene sequencing and to characterize the isolates that did not have a rep profile or had unclear banding pattern, was performed using the RAPD primer $(GTG)_5$ (5'- GTG GTG GTG GTG GTG - 3') (Wiid *et al.* 1994). PCR reactions were set up as follows: 1 µl genomic DNA (approximately 10 ng), 5 µl of each primer (5 µmol l^{-1}), 1 µl dNTPs (10 mmol l^{-1} each), 5 µl DreamTaq Green Buffer (10X) (Thermo Fisher Scientific, Waltham, MA, USA), 27.5 µl high-purity water (Purelab Flex, ELGA LabWater, Veolia Water Technologies), 5 µl DMSO, 0.5 µl DreamTaq DNA Polymerase (5 U μl^{-1}). For primer pair REP1R-I – REP2-I, the PCR program was the following: initial denaturation for 3 min at 95°C, 35 amplification cycles (30 s at 95°C, 30 s at 40°C, 4 min at 72°C), and final extension step of 10 min at 72°C. For RAPD primer $(GTG)_5$ the PCR program was the following: initial denaturation for 5 min at 94°C, 30 amplification cycles (30 s at 95°C, 1 min at 45°C, 5 min at 65°C), and final extension step of 16 min at 65°C. The PCR products were loaded on a 0.8% (wt vol^{-1}) agarose gel containing 5% ethidium bromide solution and electrophoresed for 90 min at a constant voltage of 120 V in 1X TAE (40 mmol l^{-1} Tris, 20 mmol l^{-1} acetic acid, 1 mmol l^{-1} EDTA, pH 8.3). Rep-PCR profiles were visualized with UV light and images were captured using a Molecular Imager Gel Doc XR System (Bio-Rad, Hercules, CA, USA). The profiles were analysed using the GelCompar II software (Applied Maths, Sint-Martens-Latem, Belgium). Similarities between the banding patterns were calculated using the Pearson correlation coefficient. To visualize the similarities, a dendrogram was built using the unweighted pair group method with arithmetic

means (UPGMA) algorithm. Clustering of RAPD-PCR banding patterns is shown in Figure S1 (Supporting Information).

16S rRNA gene sequencing and phylogenetic analysis

16S rRNA gene fragments were amplified using the primers 27F (5' - AGA GTT TGA TCM TGG CTC AG - 3') (Lane 1991) and 1492R (5' - CGG TTA CCT TGT TAC GAC TT - 3') (Turner *et al.* 1999). The PCR reactions were set up as follows: 1 µl genomic DNA (approximately 10 ng), 2.5 µl of each primer (5 µmol l^{-1}), 1 µl dNTPs (10 mmol l^{-1} each), 10 µl Phusion Buffer (5X) (New England Biolabs, Ipswich, MA, USA), 31 µl ELGA water, 1.5 µl DMSO, 0.5 µl Phusion DNA polymerase (2 U μl^{-1}). The PCR program was the following: initial denaturation for 30 s at 98ºC, 35 amplification cycles (10 s at 98ºC, 10 s at 53ºC, 45 s at 72ºC), and final extension step of 10 min at 72ºC. The PCR products were verified by electrophoresis on 1.5% (wt vol^{-1}) agarose gels and sent to Macrogen (Amsterdam, The Netherlands) for purification and sequencing. Raw sequence reads were trimmed and assembled in CLC Genomics Workbench software, version 5.1 (CLC Bio-Qiagen, Aarhus, Denmark). The BLAST Sequence Analysis Tool (Madden 2002), the Sequence Match Tool of the Ribosomal Database Project (RDP) (Cole *et al.* 2014) and the EzBioCloud database (Yoon *et al.* 2017) were used to assign taxonomic identity to the 16S rRNA gene sequences from the isolates. The EzBioCloud database was also used to obtain accurate similarity values between the isolates and type strains. 16S rRNA gene sequences of the unique isolates were submitted to GenBank, where they are available under accession numbers MF801315-MF801360.

Screening of isolates against pathogens

The unique strains were screened for inhibitory activity against nine pathogens: the bacteria *Staphylococcus aureus* 533R4 (DSMZ 20231) (Firmicutes), *Escherichia coli* WA321 (DSMZ 4509) (Gammaproteobacteria) and *Micrococcus luteus* ATCC49732 (Actinobacteria); the fungi *Rhizoctonia solani AG2.2 IIIB* (Basidiomycota), *Candida albicans* BSMY 212 (DSMZ 10697), *Fusarium oxyxporum* and *Beauveria bassiana* (ARSEF 2597) (Ascomycota); and the oomycetes *Pythium ultimum* P17 and *Saprolegnia diclina* (1152F4) (Heterokontophyta). The choice of these microorganisms was based on their relevance as pathogens for humans (*E.coli*, *S. aureus*, *M. luteus* and *C. albicans*), plants (*F. oxyxporum*, *R. solani* and *P. ultimum*), fish (*S. diclina*) or arthropods (*B. bassiana*).

S. aureus, *E. coli*, *C. albicans*, and *M. luteus* were grown on LB agar (Lennox LB Broth Base, agar 1.5%; Thermo Fisher Scientific, Waltham, MA, USA); *S. diclina* and *R. solani* were grown on Potato Dextrose Agar (PDA) (Potato Dextrose Broth, agar 1.5%; Sigma-Aldrich, St. Louis, MO, USA); *P. ultimum* and *F. oxysporum* were grown on 1/5 PDA ; and *B. bassiana* was grown on Sabouraud agar (SDA) (Jaronski

and Jackson 2012) with 1% yeast extract as a supplement. *S. aureus*, *E. coli* and *C. albicans* were grown at 37°C, the remaining organisms were grown at 28°C. To test the isolates against the bacteria and the fungus *C. albicans*, we used the agar overlay method. The agar plug method was used to test antimicrobial activity against the remaining fungi and against the oomycetes. For the agar overlay method, the isolates were grown overnight in Tryptic Soy Broth (TSB; Sigma-Aldrich, St. Louis, MO, USA), at 30°C with shaking at 400 rpm. Subsequently, 5 µl of liquid culture of each isolate was transferred to solid medium. Square petri dishes (120x120 mm), were used for the screening and 16 isolates were tested on each plate. *Pseudomonas protegens* Pf5, a broad range biocontrol strain, was used as a positive control on each plate (Ramette *et al.* 2011). The plates were incubated overnight at 28°C. On the same day, liquid cultures of the bacterial pathogens and of *C. albicans* were prepared by inoculating colonies in 4 ml of LB broth base (Lennox LB Broth Base, Thermo Fisher Scientific, Waltham, MA, USA) and growing them overnight at 30°C with shaking at 400 rpm. The following day, growth of the strains of interest on the agar plates was observed, and the pathogens grown during the night were prepared for the overlay. 500 µl of liquid culture of the pathogen was inoculated in 10 ml top agar (LB Broth Base, 0.7% agar), mixed well by vortexing and poured over the plate to completely cover the colonies of the isolates. The plates were then incubated overnight at the appropriate temperature for the pathogens and the next day the presence and diameter of inhibition zones were recorded. For the agar plug method, agar plugs covered in mycelium of the pathogens were placed on the plates between the isolates' colonies, after the isolates of interest had grown on the plates. The plates were sealed with parafilm and incubated at the appropriate temperature for the pathogens until the growth allowed the observation of clear inhibition zones (between three and 19 days). For both the agar overlay and the agar plug method, the screening was conducted on three different media: actinomycete isolation agar (M490), Potato Dextrose Agar (PDA) and 1/10 Tryptic Soy Agar (1/10TSA) (Tryptic Soy Broth, NaCl 0.5%, KH_2PO_4 0.1%, agar 1.5%; Sigma-Aldrich, St. Louis, MO, USA).

4.4. Results

Isolation and growth of bacteria

After dissecting the guts from the springtails *F. candida*, *F. fimetaria*, *T. minor*, *S. curviseta* and *O. cincta*, and plating them on M490 medium, colony growth was observed between day 3 and day 7. No growth was observed on negative control plates. Based on morphological differences, a total of 78 colonies were picked and transferred to TSA plates to obtain pure cultures.

Rep-PCR, RAPD-PCR and 16S rRNA gene sequencing

Genomic DNA was isolated from the pure cultures and subjected to rep-PCR to prevent further analysis of identical strains (dereplication). Isolates that produced unique molecular profiles or that did not amplify during the rep-PCR screening step, were then identified using 16S rRNA gene sequencing. Finally, RAPD-PCR was used to obtain profiles for the isolates that did not have a rep-PCR profile and possibly to differentiate between isolates with the same 16S-based identity, but obtained from different animal species. Following these molecular identification and dereplication procedures, all unique isolates were further investigated to assess their inhibitory activity against pathogens.

In total, 46 unique isolates were selected. The number of unique isolates that were obtained from each collembolan species are summarized in Table 1. Most isolates were obtained from *S. curviseta* (18) and *F. candida* (11), while only four were isolated from *O. cincta* guts. Morphological characteristics and rep- and RAPD-PCR profiles of all unique isolates are available in Table S1 (Supporting Information). Rep-PCR profiles were not obtained for 17 of the 46 unique isolates (37%), corresponding to the Firmicutes (*Staphylococcus* Fc5, Fc17c and Ff5 and *Bacillus* Sc23) some Actinobacteria (all *Streptomyces*: Fc1, Fc7, Fc12, Ff1, Ff4b Tm3, Tm6b, Sc7a and Sc8, *Glutamicibacter* Sc3 and *Cellulosimicrobium* Tm1) and some Proteobacteria (*Halomonas* Oc4 and Sc22) isolates (Table S1).

Based on their 16S rRNA gene sequence, the isolates were assigned a taxonomic identity. The top BLAST and EzBioCloud hits of the 16S rRNA gene sequences obtained from each unique isolate are represented in Table 1. Based on the comparison with type strains in the EzBioCloud database, 17 different genera were identified across the five springtail species: the Proteobacteria *Ochrobactrum* (Alphaproteobacteria, Rhizobiales), *Delftia* (Betaproteobacteria, Burkholderiales), *Stenotrophomonas*, *Pseudoxanthomonas* (Gammaproteobacteria, Xanthomonadales), *Pseudomonas, Acinetobacter* (Gammaproteobacteria, Pseudomonadales), *Serratia*, *Pantoea* (Gammaproteobacteria, Enterobacteriales) and *Halomonas* (Gammaproteobacteria, Oceanospirillales); the Firmicutes *Staphylococcus* and *Bacillus* (Bacilli, Bacillales); the Actinobacteria *Cellulosimicrobium* (Micrococcales), *Streptomyces*, *Glutamicibacter*, *Gordonia*, *Nocardioides* and *Microbacterium* (Actinomycetales). No significant differences in the occurrence of the different genera was observed between the five animal species (Chi-squared test, data not shown).

Fifteen isolates had 100% identity with their top EzBioCloud hit, 25 isolates had higher than 99% identity, and six isolates (*Streptomyces* Fc1, *Microbacterium* Fc2, *Halomonas* Oc4 and Sc22, and *Pantoea* Oc5 and Tm9b) had less than 98.65% similarity with reference sequences in EzBioCloud.

To discriminate between isolates with the same top BLAST hit, a RAPD-PCR was performed using $(GTG)_5$ primer. A profile was obtained for most of the isolates, except for Fc1 and Ff1. Isolates with similar morphologies and similar rep-PCR profile from the first screening step also showed similar RAPD-PCR profiles (see Table S1 – Supporting Information: *Pantoea* Oc5 and Tm9b; *Stenotrophomonas* Ff7b and Sc15; *Ochrobactrum* Fc6 and Ff3). In some cases, the first rep-PCR screening step was sufficient to differentiate strains (for example, compare profiles of *Microbacterium* Fc2, Fc13 and Fc16a). However, when a REP profile was not available, RAPD-PCR allowed us to differentiate between strains with similar morphology but different genotype (compare *Streptomyces* Sc7a and Sc8) or to identify additional replication among strains, which was also confirmed by the results of 16S rRNA gene sequencing. Isolates with the same EzBioCloud top hit, also had similar RAPD-PCR profiles (compare *Staphylococcus* Fc5 and Ff5); and isolates assigned by EzBioCloud to the same genus but to different species, had different RAPD-PCR profiles (compare *Microbacterium* Fc2, Fc13 and Fc16a, or *Staphylococcus* Fc5 and Fc17c). Overall, the greatest redundancy of isolates was found among the *F. candida* and *S. curviseta* isolates.

Antimicrobial screening

The antimicrobial potential of the bacterial isolates was assessed by testing their ability to inhibit growth of a variety of pathogenic microorganisms. Figure 1 shows an example of inhibition of pathogens' growth by strain *Streptomyces* Fc1 isolated from *F. candida*. The results of the inhibition assays are summarized in Table 1 and presented in more detail in Table S1 (Supporting Information). Forty-six springtail isolates were tested against nine pathogens, on three culture media, resulting in 1242 specific inhibition tests. In 222 tests (18%) inhibition was observed. As expected, the positive control *Pseudomonas protegens* Pf5 inhibited growth of all pathogens on all media. A surprisingly high number of 35 out of 46 (76%) springtail isolates inhibited at least one pathogen; 18 of these (50%) were active against both fungal and bacterial pathogens (*Streptomyces* Fc1, Fc7, Fc12, Ff1, Ff4b, Tm3, Tm6b, Sc7a and Sc8, *Stenotrophomonas* Fc3a and Ff7a, *Pseudomonas* Fc8, Oc2, Tm10a and Tm10b, *Acinetobacter* Sc11, *Serratia* Sc14, *Nocardioides* Sc24); 11 strains were active only against bacteria (*Staphylococcus* Fc5 and Ff5, *Ochrobactrum* Fc6 and Ff3, *Microbacterium* Fc16a, *Pantoea* Oc5 and Tm9b, *Cellulosimicrobium* Tm1, *Glutamicibacter* Sc3, *Acinetobacter* Sc17, *Pseudomonas* Sc19); while six were active only against fungi and/or oomycetes (*Staphylococcus* Fc17c, *Stenotrophomonas* Ff7b and Oc1, *Halomonas* Oc4, *Ochrobactrum* Tm9a and Sc9). One isolate, *Streptomyces* Sc8, inhibited the growth of all pathogens tested, like the positive control.

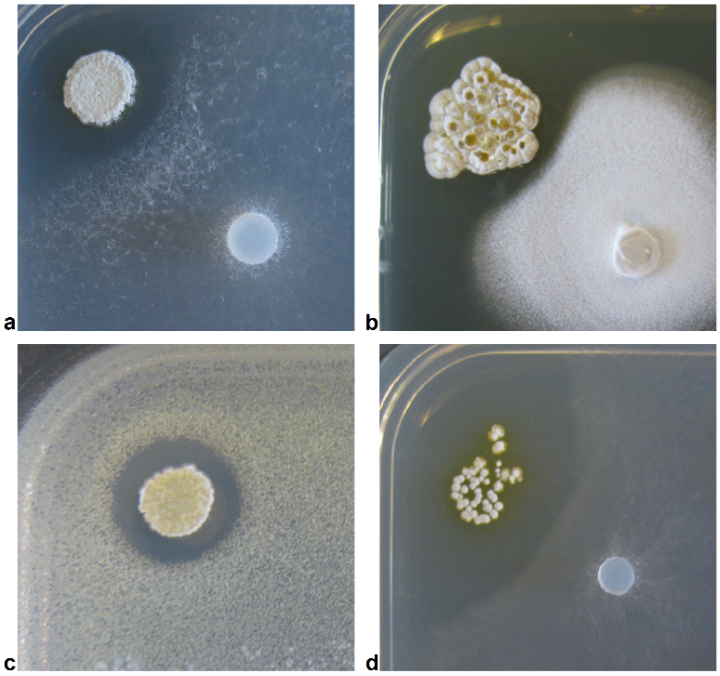

Figure 1. Pictures showing the strain *Streptomyces* sp. Fc1 (isolated from *Folsomia candida*), growing in the left upper corner, inhibiting the growth of different pathogens on a variety of media. **a**: *Fusarium oxysporum* on 1/10 TSA; **b**: *Beauveria bassiana* on M490; **c**: *Micrococcus luteus* on PDA; **d**: *Saprolegnia diclina* on 1/10 TSA.

All isolates from *F. fimetaria*, *O. cincta* and *T. minor* showed some kind of inhibitory activity (Table 1), while only 50% (nine out of 18) of *S. curviseta* isolates and 82% (nine out of 11) of *F. candida* isolates showed activity. Moreover, all of the *Streptomyces, Pseudomonas*, *Acinetobacter*, *Staphylococcus* and *Pantoea* isolates were active against some of the pathogens, regardless of the animal species from which they were derived. Finally, when considering all isolates from a springtail species, they were collectively active against all of the pathogens tested, with the exception of isolates from *O. cincta* which could not inhibit *E.coli* growth.

We observed differences in the inhibitory phenotypes between replicates of the same strains (Table S1). At the same time, patterns of inhibition were very similar for some isolates from different springtails suspected (based on 16S rRNA gene sequencing, rep- and RAPD-PCR profiles and morphologies) to belong to the same species, for example *Streptomyces* Fc7 and Ff4b, and *Ochrobactrum* Tm9a and Sc9. No significant differences in the average number of inhibitions per isolate could be observed between the five different hosts (Kruskal-Wallis test, Dunn's multiple pairwise comparison, data not shown).

Table 1. Total number of cultured isolates, top BLAST hits of the unique isolates and number of pathogens inhibited by each unique isolate for each springtail species.

Springtail	Total number of isolates growing on M490 medium	Unique isolates based on rep-PCR profiles, RAPD-PCR profiles and 16S rRNA gene sequencing						
		Isolate	Top BLAST Hit	Identity with BLAST hit	Accession BLAST hit	Top EzBioCloud hit	Identity with BLAST hit	Number of pathogens inhibited
Folsomia candida	23	FC1	*Streptomyces sp*. TTHQ-DM-1	92%	KF729623	*Streptomyces chryseus*	93.4%	8
		FC2	*Microbacterium sp*. SIB_Cu_R3	96%	KX036571	*Microbacterium yannicii*	95.9%	N.I.
		FC3a	*Stenotrophomonas sp*. MACL13A	100%	EF198252	*Stenotrophomonas maltophilia* ⑦	99.78%	4
		FC5	*Staphylococcus sp*. S07 ①	100%	FJ002588	*Staphylococcus fleurettii* ①	99.86%	2
		FC6	*Ochrobactrum sp*. MYb103 ②	100%	KX079833	*Ochrobactrum pituitosum* ②	99.85%	1
		FC7	*Streptomyces sp*. strain BV316 ③	100%	MF511802	*Streptomyces pratensis* ③	100%	5
		FC8	*Pseudomonas sp*. strain B16	100%	KY324846	*Pseudomonas koreensis*	99.71%	8
		FC12	*Streptomyces sp*. strain KIB-H0495	100%	MF164040	*Streptomyces setonii*	100%	8
		FC13	*Microbacterium sp*. T6220-7-3b strain: K13218-3-2b	100%	LC148842	*Microbacterium shaanxiense* ⑧	100%	N.I.
		FC16a	*Microbacterium natoriense* strain NW41	99%	JF915347	*Microbacterium natoriense*	99.25%	3
		FC17c	*Staphylococcus sciuri* strain FDAARGOS_285	100%	CP022046	*Staphylococcus sciuri*	99.93%	3
Folsomia fimetaria	10	Ff1	*Streptomyces sp*. strain K1	100%	KX674552	*Streptomyces rubiginosohelvolus*	100%	8
		Ff3	*Ochrobactrum sp*. JCM 28827 strain: T7426-9-3b	100%	LC150701	*Ochrobactrum pituitosum* ②	99.85%	2
		Ff4b	*Streptomyces sp*. strain BV316 ③	100%	MF511802	*Streptomyces pratensis* ③	100%	5
		Ff5	*Staphylococcus sp*. S07 ①	100%	FJ002588	*Staphylococcus fleurettii* ①	99.86%	2
		Ff7a	Uncultured *Stenotrophomonas sp*. clone OTUM16	98%	EU826667	*Stenotrophomonas terrae* ⑨	99.06%	2
		Ff7b	*Stenotrophomonas terrae* strain R-32768 ④	99%	NR_042569	*Stenotrophomonas terrae* ⑨	99.56%	4
Orchesella cincta	6	Oc1	*Stenotrophomonas sp*. LY-2	100%	LC136883	*Stenotrophomonas maltophilia* ⑦	100%	4
		Oc2	*Pseudomonas putida* strain B-18	99%	MF417798	*Pseudomonas mosselii*	99.85%	6
		Oc4	*Halomonas qiaohouensis* strain YIM QH88	99%	NR_148256	*Halomonas qiaohouensis*	98.58%	3
		Oc5	Uncultured gamma proteobacterium clone P9 ⑤	99%	KJ934770	*Pantoea dispersa* ⑤	98.64%	2

Table 1 (continued)

Tomocerus minor	**14**	Tm1	*Cellulosimicrobium funkei* strain NPZ-121T	99%	KY563746	*Cellulosimicrobium aquatile*	99.85%	1
		Tm3	*Streptomyces drozdowiczii* strain BERC6	100%	KX510091	*Streptomyces drozdowiczii*	99.7%	3
		Tm6b	*Streptomyces sp*. strain BV316 ③	100%	MF511802	*Streptomyces pratensis* ③	100%	6
		Tm9a	*Ochrobactrum sp*. strain Ktm-7 ⑥	100%	MF405118	*Ochrobactrum anthropi* ⑥	100%	1
		Tm9b	Uncultured gamma proteobacterium clone P9 ⑤	99%	KJ934770	*Pantoea dispersa* ⑤	98.64%	1
		Tm10a	*Pseudomonas sp*. strain 40	100%	KY681910	*Pseudomonas yamanorum*	99.85%	8
		Tm10b	*Stenotrophomonas maltophilia* strain S2C	100%	KT337509	*Pseudomonas beteli*	99.64%	4
Sinella curviseta	**25**	Sc1	*Pseudoxanthomonas sp*. strain Oil-1	100%	MF405126	*Pseudoxanthomonas indica*	99.64%	N.I.
		Sc3	*Arthrobacter sp*. RCB237	100%	KT260449	*Glutamicibacter soli*	99.56%	2
		Sc7a	*Streptomyces sp*. strain BV316 ③	100%	MF511802	*Streptomyces pratensis* ③	100%	7
		Sc7b	*Gordonia sp*. COL11	100%	JN695023	*Gordonia malaquae*	99.93%	N.I.
		Sc8	*Streptomyces sp*. H16 LR-2017	100%	LC230101	*Streptomyces luridiscabiei*	100%	9
		Sc9	*Ochrobactrum sp*. strain Ktm-7 ⑥	100%	MF405118	*Ochrobactrum anthropi* ⑥	100%	2
		Sc10	*Microbacterium shaanxiense* strain CCNWSP60	100%	KJ735510	*Microbacterium shaanxiense* ⑧	100%	N.I.
		Sc11	*Acinetobacter sp*. strain LSN1-10	99%	KY054572	*Acinetobacter courvalinii*	99.21%	4
		Sc14	*Serratia marcescens* strain FF6	100%	KR778806	*Serratia marcescens*	99.93%	7
		Sc15	*Stenotrophomonas terrae* strain R-32768 ④	99%	NR_042569	*Stenotrophomonas terrae* ⑨	99.49%	N.I.
		Sc16	*Delftia sp*. strain As-37	100%	MF353935	*Delftia lacustris*	100%	N.I.
		Sc17	*Acinetobacter sp*. RS206	100%	EU912468	*Acinetobacter vivianii*	99.35%	1
		Sc19	*Pseudomonas vranovensis* strain E8 - 8	100%	KY938127	*Pseudomonas donghuensis*	99.85%	3
		Sc20b	*Glutamicibacter arilaitensis* strain MLS-4-4	100%	KT997452	*Glutamicibacter arilaitensis*	99.18%	N.I.
		Sc21b	*Ochrobactrum sp*. MYb103 ②	100%	KX079833	*Ochrobactrum pituitosum* ②	99.85%	N.I.
		Sc22	*Halomonas sp*. ADMK29	98%	KU850985	*Halomonas stenophilia*	97.93%	N.I.
		Sc23	*Bacillus sp*. Ant-1b	100%	HF678912	*Bacillus weihenstephanensis*	100%	N.I.
		Sc24	*Nocardioides sp*. strain Brt-B	100%	MF405108	*Nocardioides daejeonensis*	100%	3

The numbers in circles in the “top BLAST hit” and in the “Top EzBioCloud hit” columns indicate isolates with the same top BLAST hit. E-values for the BLAST hits in the table were all lower than 1E-70. N.I. = no inhibition

Discussion

In this study, we show that bacteria isolated as cultivatable isolates from the guts of springtails are active against a broad range of microbial pathogens that are relevant for human health, agriculture and fish culturing. We identified 46 unique isolates, 35 of which (76%) showed inhibition of at least one of the pathogens tested. In principle, these isolates could serve as biocontrol agents or be further characterized for applications in the pharmaceutical sector.

Isolation of potential antimicrobial producers

With the exception of *Staphylococcus* and *Bacillus* isolates (Firmicutes), all bacteria with antimicrobial activity belong to the phyla Actinobacteria and Proteobacteria. 18 of the 46 isolates (40%) are Actinobacteria. This suggests that M490 medium can be effectively used to target this microbial group, while still allowing growth of other bacteria. Previous microbial community studies, using both culture-based and cultivation-independent approaches on eight springtail species, did not identify *Streptomyces* strains in collembolan gut (Thimm *et al.* 1998; Czarnetzki and Tebbe 2004a; Agamennone *et al.* 2015). In culture-based studies, this may have been due to specific culturing conditions, for example the use of media selecting against *Streptomyces*, or to the slow growth of Actinobacteria compared to other bacteria. In culture independent analyses, such as electron microscopy or *in situ* hybridization, underrepresentation of *Streptomyces* may be due to less optimal binding of primers, or to very low abundances of these bacteria. In contrast to *Streptomyces*, in this study we found common genera like *Stenotrophomonas*, *Ochrobactrum* and *Pseudomonas* that had been previously identified in collembolan gut (Thimm *et al.* 1998; Czarnetzki and Tebbe 2004a).

Many of the isolates did not amplify with the rep-PCR primers, which may have been due to lack of annealing of the primers with the template or to suboptimal amplification conditions. However, RAPD-PCR profiles were obtained for most isolates and were useful in discriminating between different bacterial species with the same top BLAST hit.

Activity of isolates against pathogenic fungi and bacteria

Of the 46 bacterial isolates tested, 76% showed inhibitory activity. However, pathogen inhibition was observed in only 18% of the inhibition tests performed with different combinations of isolate, pathogen and growth medium. This suggests that interactions between microorganisms are specific, and that inhibition and competition may be modulated by environmental factors (Hoek *et al.* 2016).

In this study, we confirm the broad inhibitory activity of *Streptomyces*, a genus of Gram-positive bacteria well known for the production of secondary metabolites including antibiotics (de Lima Procópio *et al.* 2012). Three *Pseudomonas* isolates

also showed broad inhibitory activity, inhibiting almost all pathogens. Furthermore, all *Acinetobacter*, *Staphylococcus* and *Pantoea* isolates showed inhibitory activity. These bacteria have previously been shown to inhibit pathogens. *Pseudomonas* species are known for their antagonistic activity against plant and fish pathogens (Berg *et al.* 2005; Liu *et al.* 2015b). *Acinetobacter* strains were previously shown to inhibit *Ralstonia solanacearum*, a plant pathogen causing bacterial wilt in tomato (Xue *et al.* 2009). The growth-inhibiting activities exerted by *Serratia marcescens* have also been observed before, and this species is known to synthesize the red antimicrobial compound prodigiosin (Suryawanshi *et al.* 2016).

Most likely, the observed inhibitions by the gut isolates are due to the production of antimicrobials (Makras and De Vuyst 2006). Microorganisms can use such compounds to compete with other species or with individuals of the same species (Hibbing *et al.* 2010). Competition can be particularly intense in environments such as the rhizosphere and the soil ecosystem in general. Here, complex interactions between plant roots, pathogenic and beneficial microorganisms take place, driving a complex warfare based on the production of chemical toxins and on the evolution of strategies to resist them (Raaijmakers *et al.* 2009).

Springtails can be particularly active in the rhizosphere of plants, where they are attracted by high microbial activity and biomass and they may establish symbiosis with microorganisms (Endlweber, Ruess and Scheu 2009). In the rhizosphere, collembolans feed on a diversity of fungi, including arbuscular mycorrhizal (AM), saprotrophic and pathogenic ones (Broza, Pereira and Stimac 2001; Jonas *et al.* 2007). For example, the root pathogens *F. culmorum* and *R. solani* constitute high quality food sources for *F. candida* and *F. fimetaria* (Larsen *et al.* 2008). *F. fimetaria* not only feeds on *R. solani*, but is also effective in reducing infection by this fungus in soil under experimental conditions (Lootsma and Scholte 1997). Moreover, *S. curviseta* was observed to graze on *F. oxysporum* (Nakamura, Matsuzaki and Itakura 1992), while *F. candida* is known to feed on *F. solani* and is even reproductively active on it (Bastian *et al.* 2010). These observations suggests that springtails may be beneficial for plant health, by using plant pathogens as food source. At the same time, microorganisms associated with springtails may respond with antimicrobial production to the presence of pathogens in their environment. For example, we observed that *Streptomyces* and *Pseudomonas* isolated from springtails were active against *R. solani*, *P. ultimum* and *F. oxysporum*, plant pathogens that mainly infect plant roots.

Antimicrobial compounds produced by springtails' isolates could be used as biocontrol agents. In the past, biocontrol agents have been obtained from bacteria isolated from the rhizosphere based on their antagonistic activity against pathogens (Trotel-Aziz *et al.* 2008). Compounds with antibacterial and antifungal activity may also find applications in the pharmaceutical and medical sector. Many of the

antibacterial and antifungal products in use today are natural products. The majority of known antibiotics, such as streptomycin, tetracycline and chloramphenicol, are produced by *Streptomyces*, a genus of soil-living bacteria that has been a source of antimicrobial compounds for decades and continues to be of interest for drug discovery nowadays (Antoraz *et al.* 2015). The most famous antibiotic and the first to be described, penicillin, is produced by a fungus. And filamentous fungi, especially the genera *Penicillium* and *Aspergillus*, also provide promising candidate for the development of antifungal therapies (Garrigues *et al.* 2017).

Using methods that aimed to enrich for antimicrobial production, we found higher levels of inhibitory activity compared to those previously found in soil bacteria. For example, one study found that 20 out of 62 soil strains (32%) had moderate to high activity against *B. subtilis*, *S. aureus*, *E. coli* and *C. albicans* (Singh *et al.* 2009), while another found that only 10 strains out of 160 isolated from soil showed antifungal activity against plant pathogens (Petatán-Sagahón *et al.* 2011). Other studies have found high levels of antimicrobial activity in bacteria associated with both marine (Zheng *et al.* 2000; Yung *et al.* 2011) and terrestrial invertebrates (Fredenhagen *et al.* 1987; Piel *et al.* 2005), suggesting that microbial communities in invertebrate guts may constitute important targets for the discovery of new therapeutic agents. Whether host-associated microbial communities are enriched for antimicrobial functions compared to the free-living ones in water and soil environments is an interesting question that could be addressed by future comparative studies.

Ecological relevance of animal-microbe associations

One might argue that soil invertebrates, living in a microbe-dominated environment, would benefit from antimicrobial activity in their gut, however, care must be taken in assuming benefits or even coevolution when there is in fact no evidence (Moran and Sloan 2015). Antimicrobial biosynthesis is often triggered by interactions and signaling between microorganisms (Tyc *et al.* 2014) or by the presence of specific elicitors in the environment (Rigali *et al.* 2008). In some cases, antimicrobial production may result in indirect benefits for the host by conferring protection against predators and parasites. For example, fungus-growing ants harbour *Streptomyces* bacteria that produce antibiotics specifically targeting the parasitic fungus *Escovopsis*. As a result, the ants and their gardens are protected from fungal infection (Haeder *et al.* 2009). Another example is provided by pederin, a polyketide produced in the beetle *Paederus fuscipes* by its symbiont *Pseudomonas aeruginosa*. Pederin functions as a chemical defense and protects the larvae of the beetle from predatory spiders (Kellner and Dettner 1996; Piel *et al.* 2005). Similarly, in larvae of the marine bryozoan *Bugula neritina* bryostatins, polyketide metabolites produced by the symbiont *Endobugula sertula*, confer protection from predators (Lopanik, Lindquist and Targett 2004). Also among vertebrates there are examples of host-

microbe associations where antimicrobials produced by symbiotic bacteria contribute to the host fitness (Soler *et al.* 2008).

The gut of springtails has been described as a selective habitat for microorganisms (Thimm *et al.* 1998). It is possible that antimicrobials affect microbial community dynamics in this habitat, by regulating the microbial density or by inhibiting pathogenic microorganisms. It has been shown that entomopathogenic fungi (EPF), used as microbial control agents against soil pests (Erler and Ates 2015), are of low virulence to springtails (Broza, Pereira and Stimac 2001; Dromph and Vestergaard 2002) as compared to target hosts (Zimmermann 2007). For example, collembolans are highly resistant against *B. bassiana*, a fungus parasitic to a wide range of arthropods such as ants and beetles (Zimmermann 2007). *B. bassiana* has been found on the surface and in the guts of collembolans (Greif and Currah 2007; Anslan, Bahram and Tedersoo 2016). In this study, we isolated bacteria inhibiting *B. bassiana* from all springtail species. However, whether antimicrobials are produced in the guts of springtails, and whether they are active against pathogenic microorganisms in that environment, still needs to be investigated. Susceptibility tests with germ-free animals may provide clues about possible roles of microbes and microbial products in pathogen defense.

Future directions

This study has identified bacterial isolates that inhabit the gut of springtails and that are able to inhibit pathogenic microorganisms. Six of these isolates share less than 98.65% similarity to type strains in EzBioCloud. Although 16S rRNA gene sequence similarity suggests that these isolates may constitute novel species (Kim *et al.* 2014), this possibility should be confirmed by more extensive sequencing and phenotypic characterization. Of these six isolates, four were active against pathogens. This suggests that soil invertebrate guts are underexplored environmental niches that have a potential for the discovery of new microbial species and, in turn, new antibiotics (Taylor 2013).

Following these findings, a good strategy to identify molecules with interesting properties would be to extend the functional screens like the one performed in this study. For example, pathogenic strains known to activate specific secondary metabolism pathways could be included in the inhibition assays. Also, focus could be placed on the isolates that showed broad inhibitory activity against pathogens. Promising strains from this study, for example, would be *Streptomyces* Sc8, Fc12, Ff1 and Tm6b, and *Pseudomonas* Fc8 and Tm10a, all showing a broad spectrum of pathogen inhibition. These strains could be further characterized to identify the molecular basis of inhibition. Such studies are underway in our group. For instance, from a previous survey of *F. candida*'s gut, we isolated a *Bacillus toyonensis* (VU-DES13, Janssens *et al.* 2017) showing a broad spectrum of pathogen inhibition. We subsequently harvested from this isolate an antimicrobial fraction inducing cell wall

stress activity (as revealed by a reporter assay) and inhibiting the growth of the fungus *C. albicans* (Agamennone *et al.* unpublished).

In addition to functional studies, metagenomic profiling of complex microbial communities can provide clues for potentially useful catalytic functions. Recently, we sequenced the functional metagenome of *F. candida*'s gut, and we are currently exploring the annotations which may be associated with antibiotic biosynthesis. Cloning and functional screening of these genes can integrate metagenomic information and potentially lead to antimicrobial compound discovery.

To our knowledge, this is the first study to investigate antimicrobial potential in springtails' gut microbial communities. We showed that bacteria isolated from the gut of springtails inhibit a variety of pathogens, a possible sign of antimicrobial production. Although the gut microbial communities of the springtails studied here show a clear potential for drug discovery, they also likely constitute the tip of the iceberg in this regard. Antimicrobial compounds have been previously isolated from microbes associated with different invertebrate hosts, suggesting that host-associated microbial communities are rich sources of antimicrobials in general. This is likely the result of natural interactions between resident and pathogenic microorganisms, leading to antimicrobial warfare within the habitat constituted by the host. Further investigations should aim to explore the potential of gut microbiota as a source of novel antimicrobials, identify targets of interest for the pharmaceutical industry and for agricultural applications, and understand the ecological relevance of antimicrobial production for springtails and other soil invertebrates.

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Supplementary Information

Figure S1: Clustering of RAPD-PCR banding patterns of bacterial isolates obtained from springtails

Table S1: Morphological and molecular characterization and pattern of pathogen inhibition of the unique bacterial strains isolated from springtails' guts (available at the online version of this paper)

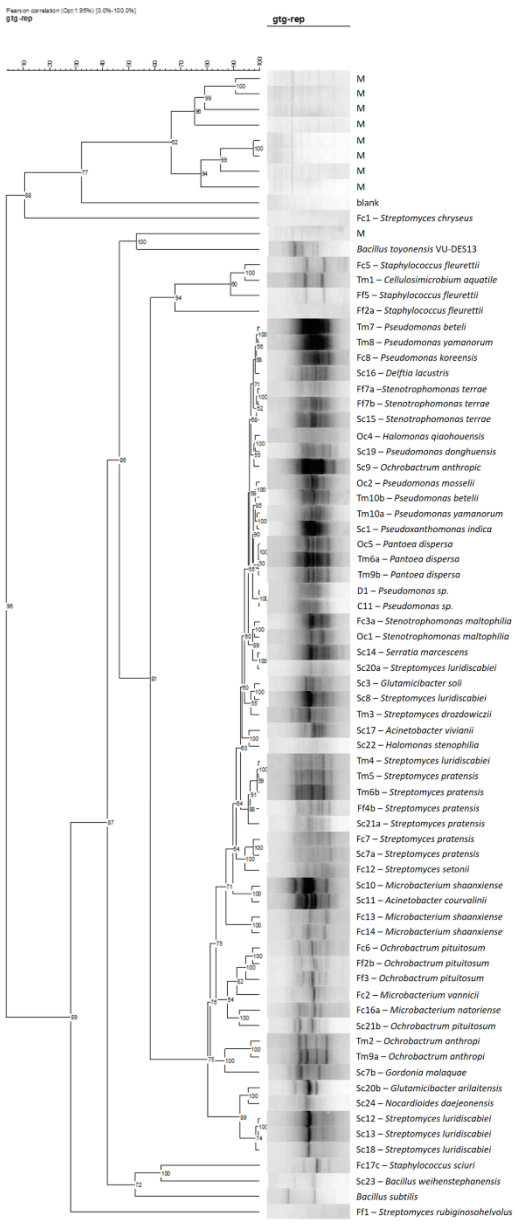

Figure S1. Clustering of RAPD-PCR banding patterns of bacterial isolates obtained from springtails. The profiles were obtained through RAPD-PCR with $(GTG)_5$ primer. The dendrogram was obtained through UPGMA-based cluster analysis. Cophenetic correlation coefficients are indicated at the root of the nodes on the dendrogram. The cluster contains the RAPD profiles of 60 isolates that had previously been selected based on their rep-PCR profiles. The names of the isolates in the cluster correspond to the top EzBioCloud hits. The cluster also contains markers (indicated with the letter M), a blank, and the RAPD-PCR patterns of four other isolates used as additional references (a *Bacillus subtilis*, and three strains that had been previously isolated from *F. candida*: *B. toyonensis* VU-DES13, *Pseudomonas* D1 and *Pseudomonas* C11).